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## The location of redox-sensitive groups in the carrier protein of proline at the outer and inner surface of the membrane in *Escherichia coli*

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Evidence is presented in this report for the presence of two sets of dithiols associated with proline transport activity in *Escherichia coli*. One set is located at the outer surface, the other at the inner surface of the cytoplasmic membrane.

Treatment of right-side-out membrane vesicles from *E. coli* ML 308-225 with the membrane-impermeable oxidant ferricyanide resulted in inhibition of L-proline uptake without having significant effect on the magnitude of the  $\Delta\tilde{\mu}_{H^+}$ . Subsequent addition of reducing agents restored proline transport activity. The membrane-impermeable SH-reagent glutathione hexane maleimide inhibited proline transport in right-side-out membrane vesicles irreversibly. Pretreatment of the vesicles with ferricyanide protected the carrier against inactivation by glutathione hexane maleimide.

Electron transfer in the respiratory chain of right-side-out vesicles led to the generation of a  $\Delta\tilde{\mu}_{H^+}$ , interior negative and alkaline, and the conversion of a disulphide to a dithiol in the proline carrier as is shown by the increased inhibition of proline transport by the membrane impermeable dithiol reagent 4-(2-arsonophenyl)azo-3-hydroxy-2,7-naphthalene disulphonic acid (thorin). The inhibition exerted by thorin was completely reversed by dithiothreitol. Pretreatment of the vesicles with thorin protected against glutathione hexane maleimide inhibition, indicating that both reagents react with the same group.

Treatment of inside-out membrane vesicles with ferricyanide inactivated the proline transport system reversibly. The oxidizing effect of ferricyanide in inside-out vesicles resulted in protection against inhibition by glutathione hexane maleimide. Imposition in these vesicles of a  $\Delta\tilde{\mu}_{H^+}$ , interior positive and acid, also protected the proline carrier against glutathione hexane maleimide inactivation, indicating that a dithiol is converted to a disulphide upon energization.

Sulphydryl groups play an important role in the function of many bacterial, chloroplast and mitochondrial transport and energy transducing systems [1–6]. Many of these membrane-bound proteins contain dithiol groups which could play a role in transport and other membrane related processes [7–14].

Recently we presented evidence that dithiol-disulphide interchanges effect the activity of three different transport systems: the *Escherichia coli* phosphoenolpyruvate-dependent glucose transport system [15] and the *E. coli* proton-symport transport systems for proline and lactose [16]. The reduced dithiol form has a low  $K_m$  for solute and the oxidized disulphide has a high  $K_m$ . The changes between the oxidized and reduced states can be generated either by artificially changing the redox potential with oxidizing and reducing agents or by establishing a  $\Delta\tilde{\mu}_{H^+}$  across the membrane. On the basis of these observations and of similar reports on other systems we proposed that dithiol-disulphide interchange could play a general role in transport and energy-transducing processes [17]. One element of this hypothesis is that dithiols and disulphides are located at different positions in the membrane and experience changes in oxidation state in response to a  $\Delta\tilde{\mu}_{H^+}$ . In our previous studies

membrane-permeable sulphydryl reagents were used and no conclusion could be drawn about the location of the redox-sensitive groups. In the present study we have used membrane-impermeable reagents. The results demonstrate the presence of two sets of dithiols in the proline carrier. One dithiol is located at the outer surface, the other at the inner surface of the membrane. Their oxidation state can change in response to a  $\Delta\tilde{\mu}_{H^+}$ .

### MATERIALS AND METHODS

#### *Growth of cells and isolation of membrane vesicles*

*Escherichia coli* ML 308-225 ( $i^-$ ,  $z^-$ ,  $y^+$ ,  $a^-$ ) were grown on minimal medium A [18] with 1% sodium succinate and 0.1% yeast extract. Right-side-out membrane vesicles were prepared as described by Kaback [19]. Inside-out membrane vesicles were obtained from cells harvested in the late exponential phase of growth. The cells were washed twice with 100 mM potassium phosphate, pH 6.6 and 10 mM potassium ethylenediaminetetraacetate and resuspended in the same buffer at a final concentration of 0.2 g of wet cells/ml. The cells were lysed by a single passage through a french press cell at 51.75 MPa at 4 °C under nitrogen. The suspension was centrifuged at  $50\,000 \times g$  for 20 min. The supernatant solution was centrifuged for 1 h at  $150\,000 \times g$ . The pellet was washed once with 100 mM potassium phosphate, pH 8.0 and 10% (v/v) glycerol, and resuspended in the same buffer to 15–20 mg of protein/ml.

*Abbreviations and trivial names.* PMS, phenazine methosulphate; MalNEt, N-ethylmaleimide; GSM, glutathione hexane maleimide; pCMBS, *p*-chloromercuribenzenesulfonic acid; diamide, diazene dicarboxylic acid bis(*N,N*-dimethyl-amide); thorin, 4-(2-arsonophenyl)azo-3-hydroxy-2,7-naphthalene disulphonic acid;  $Ph_4P^+$ , tetraphenylphosphonium ion.

### Transport assays

The uptake of L-proline L-[U- $^{14}$ C]proline, 3.5  $\mu$ M, specific activity 285 Ci/mol) in right-side-out membrane vesicles was studied under aerobic conditions at 25 °C with ascorbate (10–20 mM) and phenazine methosulphate (0.1 mM) or D-lactic acid (20 mM) as electron donors as described [20]. The initial rates of uptake were the values obtained 15 s after the addition of the solute. Each value reported is an average of three measurements. The uptake of L-proline in right-side-out and inside-out membrane vesicles, energized by a chlorate diffusion potential, was performed by diluting 10  $\mu$ l of the membrane vesicle suspension (15–20 mg protein/ml) into 100  $\mu$ l 100 mM choline-Hepes, 40 mM choline chlorate, pH 8.0 and 3.5  $\mu$ M L-[ $^{14}$ C]proline at 25 °C. In control experiments, no chlorate gradient, the concentrated vesicle suspension was first equilibrated with 40 mM choline chlorate for 15 min before dilution.

### Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was performed as described [21]. The peak areas were estimated from the immunoprecipitates of the ATPase and the NADH dehydrogenase that were stained either with Coomassie brilliant blue or by zymogram techniques.

### Measurements of $\Delta\psi$

The  $\Delta\psi$  was calculated from the distribution of tetraphenylphosphonium ( $\text{Ph}_4\text{P}^+$ ) between the bulk phase of the external medium and the intravesicular fluid. The concentration of  $\text{Ph}_4\text{P}^+$  in the external medium was determined with a  $\text{Ph}_4\text{P}^+$ -selective electrode constructed according to Shinbo et al. [22]. The intravesicular concentration was calculated from the amount of  $\text{Ph}_4\text{P}^+$  which had disappeared from the external medium and was accumulated in the intravesicular space (2.2  $\mu$ l/mg membrane protein [23]). An attempt was made to correct for  $\text{Ph}_4\text{P}^+$  binding by subtracting the amount of probe bound under deenergized conditions from the total amount of probe taken up under energized conditions [24].

### Measurements of oxygen uptake

The rates of oxygen uptake were measured as described previously [25]. Assay mixtures (2.0 ml) contained 50 mM potassium phosphate, pH 7.0; 10 mM magnesium sulphate; 0.6 to 1.0 mg membrane protein and 20 mM lithium D-lactate.

### Protein determination

Protein was measured according to Lowry et al. [26] using bovine serum albumin as standard.

### Materials

Glutathione hexane maleimide was synthesized and characterized according to a published procedure [27]. 4-(2-Arsonophenyl)azo-3-hydroxy-2,7-naphthalene disulphonic acid was purchased as thorin from Ventron Corporation and used without further purification. Radioactive proline (specific activity 285 Ci/mol) was purchased from the Radiochemical Centre, Amersham). All other chemicals were reagent grade.

## RESULTS

### Effect of ferricyanide on solute transport in right-side-out vesicles

The uptake of proline by *Escherichia coli* membrane vesicles energized by D-lactate oxidation (Fig. 1) or by an

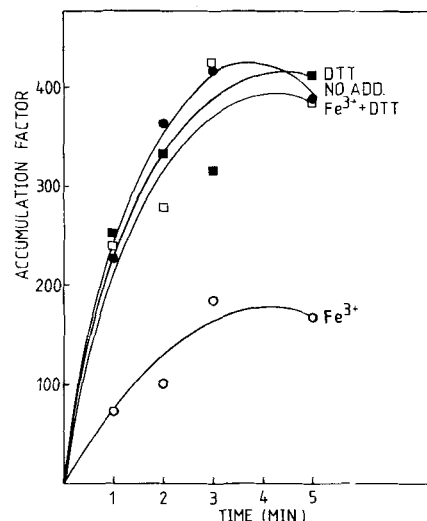


Fig. 1. Effect of ferricyanide and dithiothreitol (DTT) or L-proline uptake in right-side-out membrane vesicles. Vesicles (13 mg membrane protein/ml) were preincubated with 10 mM ferricyanide or 20 mM dithiothreitol on ice 60 min. Subsequently the vesicles were diluted to 1.3 mg membrane protein/ml with 50 mM potassium phosphate, pH 7.0 and 10 mM magnesium sulphate containing 10 mM ferricyanide or 20 mM dithiothreitol. After 5 min incubation at 26 °C, 20 mM lithium D-lactate was added as electron donor and uptake was studied as described in Materials and Methods. Dithiothreitol (20 mM) addition after ferricyanide treatment was made just prior to the additions of L-[ $^{14}$ C]proline

artificially imposed chlorate diffusion potential (data not shown) was strongly inhibited by 10 mM ferricyanide, a membrane impermeable oxidant. In the presence of excess dithiothreitol or other reductants the inhibition exerted by ferricyanide was completely released. The inhibition by ferricyanide could not be explained by a decreased flow of electrons to oxygen and consequently a decreased proton-motive force. In the presence of 10 mM ferricyanide the rate of oxidation of D-lactate was inhibited by only 15% and the  $\Delta\psi$  was decreased from  $-124$  mV to  $-113$  mV. The  $\Delta\psi$  was measured at pH 8.0, where the  $\Delta\text{pH}$  is negligible and the  $\Delta\psi$  is essentially the only component of the  $\Delta\mu_{\text{H}^+}$  [28, 29]. Ferrocyanide (up to 10 mM) had no inhibitory effect on proline uptake (data not shown).

### A thiol-containing redox center in the proline carrier on the outer surface is oxidized by ferricyanide

The data presented above indicate that the inhibition exerted by ferricyanide was the result of an effect of the redox potential of the external medium on the redox state of a redox couple on or near the outer side of the membrane.

Support for the involvement of thiol groups in the proline carrier at the outer surface of the membrane has been supplied by studies with the membrane impermeable SH reagent glutathione hexane maleimide. Glutathione hexane maleimide irreversibly inhibits ascorbate/PMS-driven proline transport by the membrane vesicles [1]. Pretreatment of the vesicles with ferricyanide in the absence of electron donor protects against glutathione hexane maleimide inhibition of proline transport. This is shown in Fig. 2 for proline transport energized by an artificially imposed  $\Delta\psi$ . Similar results were obtained for proline transport energized by ascorbate-PMS oxidation (data not shown).

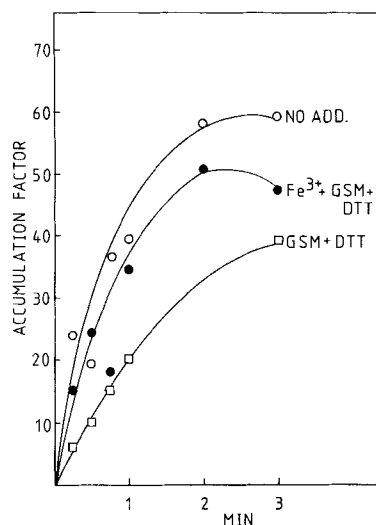


Fig. 2. Protection of ferricyanide against glutathione hexane maleimide inactivation. Right-side-out membrane vesicles (15 mg membrane protein/ml) were incubated with 4 mM GSM on ice for 20 min. If ferricyanide was added to protect against GSM inactivation the vesicles were first preincubated for 60 min on ice with 10 mM ferricyanide. The reactions of ferricyanide and GSM were stopped by the addition of 20 mM dithiothreitol (DTT). After 30 min incubation with dithiothreitol the vesicles were energized by a chlorate diffusion potential as described in Materials and Methods

#### The redox centre of the proline carrier contains a dithiol group

Inhibition of enzymes by arsine oxides is generally accepted as strong evidence for the presence of vicinal sulphhydryl groups [30]. Inhibition involves the formation of a cyclic dithiol arsenite whose stability is determined partly by a proper juxtapositioning of the thiols. In our earlier study [16] we showed that phenylarsine oxide reversibly inhibited proline transport. This reagent, however, is membrane-permeable. The highly polar arsonic acid, thorin, should be membrane-impermeable and for this reason was employed to help determine whether it is a dithiol at the outer surface which ferricyanide protects from reaction with glutathione hexane maleimide.

Thorin treatment of the membrane vesicles energized by ascorbate-PMS or D-lactate oxidation led to a 60% inhibition of proline uptake (Fig. 3). Treatment of the membrane vesicles with thorin prior to energization led to a slight inhibition of proline transport.

Incubation of membrane vesicles with thorin in the presence and in the absence of a  $\Delta\mu_{H^+}$  was carried out just prior to the measurement of the initial rate of proline uptake. In contrast to thiol reagents, dithiol reagents have essentially no effect on D-lactate oxidation as was shown by oxygen consumption experiments (data not shown).

These results indicate that a dithiol in the proline carrier becomes accessible at the outside of the membrane in response to a  $\Delta\mu_{H^+}$ , interior negative and alkaline.

#### Protection of thorin against GSM inhibition

To determine whether the site which irreversibly reacts with GSM is the same as the dithiol which complexes with thorin, the protection of proline transport activity against GSM inactivation was examined (Fig. 4).

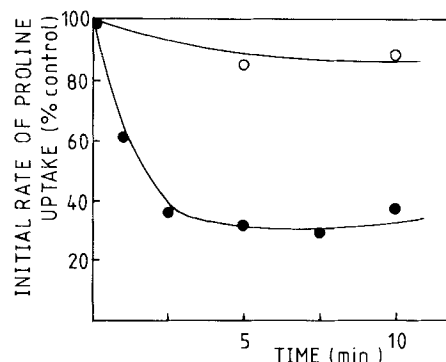


Fig. 3. Effect of thorin in the presence and the absence of the  $\Delta\mu_{H^+}$  on proline transport in right-side-out membrane vesicles. Vesicles suspended to 1.4 mg membrane protein/ml in 50 mM potassium phosphate pH 7.0 and 10 mM magnesium sulphate were incubated under aerobic conditions with 1 mM thorin in the presence (●) or in the absence (○) of 20 mM ascorbate + 100  $\mu$ M PMS at room temperature. At the times indicated the initial rate of proline uptake was determined. In the case of thorin inactivation in the absence of a  $\Delta\mu_{H^+}$ , ascorbate + PMS was added just prior to the addition of L-[ $^{14}$ C]proline

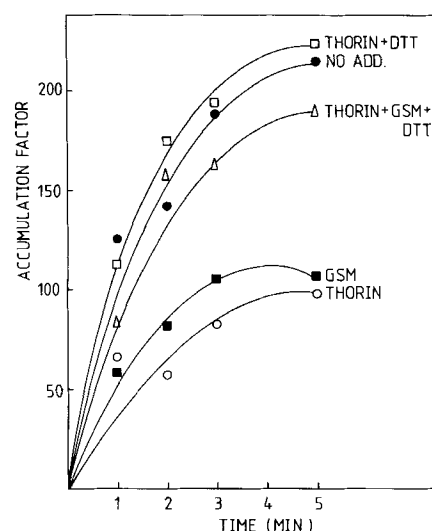


Fig. 4. Protection of thorin against glutathione hexane maleimide inactivation. Right-side-out membrane vesicles were diluted to 1.4 mg membrane protein/ml with 50 mM potassium phosphate pH 7.0 and 10 mM magnesium sulphate. Membrane vesicles were incubated under aerobic conditions in the presence of an electron donor with 1 mM thorin for 5 min at 26°C and, if indicated, subsequently with 5 mM GSM for another 5 min. Ascorbate-PMS (20 mM and 0.1 mM final concentrations, respectively) were added 30 s prior to the addition of the sulphhydryl reagents. Where indicated 10 mM dithiothreitol (DTT) was added to stop the reaction of GSM, or release the inhibition of thorin. When sequential additions are involved the order of addition is indicated by the order listed in the figure. Uptake of L-[ $^{14}$ C]proline was started immediately after the end of the reactions

Exposure of vesicles to 5 mM GSM or 1 mM thorin for 5 min in the presence of  $\Delta\mu_{H^+}$  resulted in 50% inhibition of proline transport. The thorin inhibition but not the GSM inhibition could be relieved by subsequent treatment with dithiothreitol before measuring transport. When thorin was added prior to GSM virtually complete protection against GSM inhibition was found. These data and those of the previous sections supply strong evidence that dithiol groups

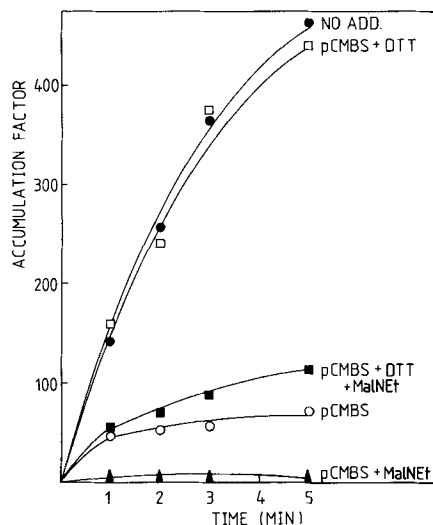


Fig. 5. The effect of pCMBS, MalNet and dithiothreitol (DTT) of proline uptake in right-side-out membrane vesicles. The vesicles (12 mg membrane protein/ml) were incubated at room temperature for 90 min with 200  $\mu$ M pCMBS and, where indicated, for an additional 20 min with 500  $\mu$ M MalNet. Dithiothreitol (10 mM) was added to stop the reaction of MalNet, or release the inhibition by pCMBS. When sequential additions are involved the order of additions is indicated by the order listed in the figure. Immediately after the end of the reactions the vesicles were diluted to 1.0 mg membrane protein/ml in 50 mM potassium phosphate, pH 7.0 and 10 mM magnesium sulphate. Aerobic uptake was executed as described under Materials and Methods with ascorbate-PMS (10 and 0.1 mM final concentrations, respectively) as the electron donor

associated with the proline carrier become accessible to the outer side of the membrane when a  $\Delta\mu_{H^+}$  is established.

*The involvement of a functional thiol group in the proline carrier, not located on the outer surface*

Evidence for at least one functional thiol in the proline transport system, not located on the outer surface is presented in Fig. 5. pCMBS is a membrane impermeable reagent whose reaction with thiols can be reversed by addition of excess dithiothreitol or mercaptoethanol [4]. MalNet on the other hand is membrane permeable and reacts irreversibly with SH groups. Our previous studies showed that 500  $\mu$ M MalNet completely inactivated proline transport [16]. The data in Fig. 5 indicate that exposure of vesicles to 200  $\mu$ M pCMBS for 90 min resulted in 85% inhibition of ascorbate/PMS-driven L-proline uptake but that this inhibition could be completely reversed by dithiothreitol. Treatment of vesicles with pCMBS (200  $\mu$ M) and subsequently with MalNet (500  $\mu$ M) resulted in a complete inhibition of proline transport but this inhibition could be only partially reversed by dithiothreitol. If the membrane impermeable GSM (10 mM) was used instead of MalNet after the 60 min pCMBS treatment, the inhibition was completely reversed by dithiothreitol. These data indicate that pCMBS protects the proline carrier on the outer side of the membrane against MalNet or GSM inactivation. This is in agreement with the previously reported high stability of the complexes of methyl-Hg<sup>2+</sup> reagents and SH groups [31]. The inhibition obtained with pCMBS + MalNet after dithiothreitol treatment must therefore be caused by the MalNet reaction with thiol groups in the proline carrier not located near the outer surface.

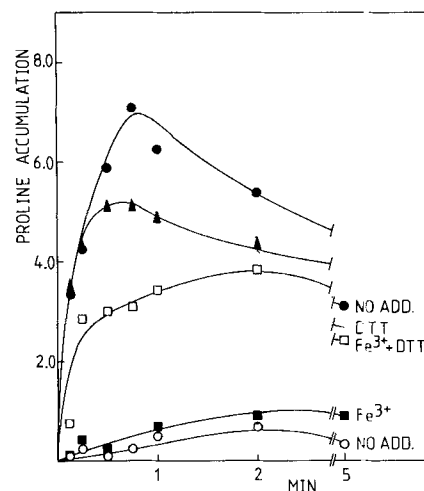


Fig. 6. Effect of ferricyanide and dithiothreitol on L-proline uptake in inside-out membrane vesicles. Vesicles (16.5 mg membrane protein/ml) were incubated with 10 mM ferricyanide and 20 mM dithiothreitol on ice for 60 and 30 min, respectively. When sequential additions were involved the order of additions is indicated by the order listed in the figure. For uptake of L-proline in response to a chlorate diffusion gradient the vesicles were diluted in buffer with 10 mM ferricyanide and/or 20 mM dithiothreitol as described in Materials and Methods. Control uptake in response to a chlorate gradient (●) or in the absence of a chlorate gradient (○) was executed in the same way, except that the redox mediators were omitted from the buffer

*Effects of ferricyanide and membrane impermeable (di)thiol reagents on proline transport in inside-out membrane vesicles*

In order to investigate more directly the involvement of redox-sensitive dithiol groups on the inner surface of the membrane, the effects of ferricyanide, GSM and thiorin on proline transport in inside-out membrane vesicles were studied. The orientation of the vesicle preparations was less than 10% right-side-out as was shown by immuno absorption experiments of the ATPase and the NADH-dehydrogenase (data not shown).

The inside-out membrane vesicles accumulated L-proline in response to an artificially imposed chlorate diffusion potential, inside negative (Fig. 6). This transient proline accumulation was almost completely inhibited by 10 mM ferricyanide. Addition of excess dithiothreitol after ferricyanide treatment released this inhibition to a large extent. These observations indicate that a redox-sensitive group in the proline carrier is located at the inner surface of the cytoplasmic membrane. The nature of this redox-sensitive group was studied.

The impermeable thiol reagent, glutathione hexane maleimide (2 mM), inactivated the proline carrier in the inside-out vesicles irreversibly (Fig. 7). Pretreatment of the vesicles with 5 mM or 10 mM ferricyanide protected almost completely the proline transport system against GSM (2 mM) (data not shown).

The results indicate that the proline carrier possesses a redox-sensitive dithiol group on the outer surface of the inside-out membrane vesicles.

*$\Delta\mu_{H^+}$  protects proline transport against GSM inhibition*

Redox-sensitive groups at the outer surface of the membrane will become more reduced with respect to identical groups located at the inner surface [17] when a proton-motive

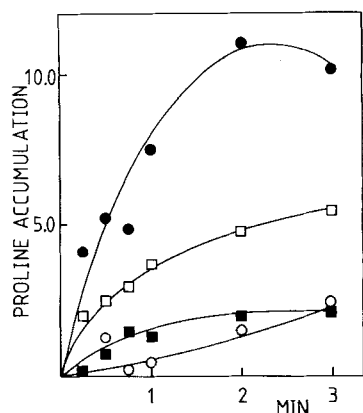


Fig. 7. Effect of  $\Delta\mu_{H^+}$  on GSM inactivation of proline transport in inside-out membrane vesicles. For GSM inactivation in the presence (□) or in the absence (■) of 20 mM ascorbate and 0.1 mM PMS. The vesicles were diluted to 2.8 mg/ml with 100 mM choline-Hepes, pH 8.0. After 2 min incubation at 25°C under a water saturated oxygen atmosphere GSM was added to a final concentration of 2 mM. After another 10 min the reaction of GSM was stopped by the addition of 10 mM dithiothreitol. Uptake of L-proline was started after 30 min by the addition of 40 mM choline chlorate, pH 8.0 and 3.5  $\mu$ M L-[ $^{14}$ C]proline to the vesicle suspension. Control vesicles were treated in the same way, except that GSM and dithiothreitol were omitted from the incubation buffer. Uptake of L-proline was executed in the presence (●) and in the absence (○) of a chlorate diffusion potential

force inside negative and alkaline is imposed across the membrane and more oxidized if a reversed  $\Delta\mu_{H^+}$  is imposed. Addition of ascorbate-PMS to inside-out membrane vesicles results in a  $\Delta\mu_{H^+}$ , inside positive and acid. Fig. 7 shows that the inhibitory action of glutathione hexane maleimide is reduced under these conditions. The results demonstrate that a reversed membrane potential decreases the accessibility of thiol groups in the proline carrier on the outer surface of the inverted membrane vesicle.

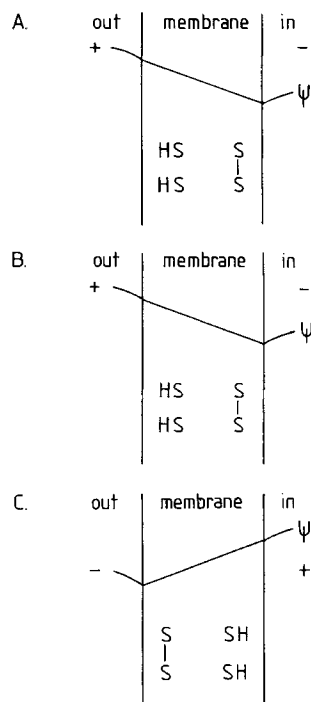
## DISCUSSION

The proline and lactose transport activities of *Escherichia coli* right-side-out cytoplasmic membrane vesicles are more sensitive to inhibition by SH reagents when the membranes are energized by a  $\Delta\mu_{H^+}$ , interior negative and alkaline [1]. Similar observations have been published for a number of other bacterial and mitochondrial  $\Delta\mu_{H^+}$ -dependent transport systems. Our recent investigations of the proline and lactose transport systems have shown that the  $\Delta\mu_{H^+}$ -dependent changes in SH-group reactivity parallel changes in the  $K_m$  for solute binding in both systems. Moreover, these same changes can be generated by artificially altering the redox state of the carrier using oxidizing or reducing agents. From these data we have concluded that the redox related conversion which alters the SH-group reactivity and the  $K_m$  for solute binding is a dithiol-disulphide interconversion.

Kaback and Patel demonstrated the SH-group sensitivity of the lactose and proline carrier by treating cytoplasmic membrane vesicles with diamide, a reagent which can either alkylate mono-thiols or catalyze the conversion of dithiols to disulphides [2]. Cohn et al. [1] treated cytoplasmic membrane vesicles with glutathione hexane maleimide in order to locate the sensitive SH groups. Since GSM inhibited when added to the exterior of the vesicles they concluded that the SH group must be located at or near the outer surface.

In this publication we have shown that the SH groups reacting at the outer surface are dithiols rather than monothiol since non-permeant oxidizing agents which convert dithiols to disulfides protect against inhibition by GSM as do arsenicals which protect the dithiols by forming cyclic dithiol arsenite complexes.

In previous investigations of the *E. coli* phosphoenolpyruvate-dependent glucose transport system [15, 32], using inverted cytoplasmic membrane vesicles we observed that a dithiol on the inner surface of the cytoplasmic membrane could be protected from reaction with SH reagents in three ways: (a) by preincubation with arsenicals; (b) by preincubation with oxidizing agents; (c) by establishing a  $\Delta\mu_{H^+}$  over the membrane using oxidizable substrates such as reduced PMS. The last two procedures presumably led to the conversion of the dithiol to a disulfide. This effect of the  $\Delta\mu_{H^+}$  is the opposite of that observed in the right-side-out vesicle studies reported above where the more rapid inactivation in the presence of a  $\Delta\mu_{H^+}$  would be consistent with the  $\Delta\mu_{H^+}$ -dependent conversion of a disulfide to a dithiol. The opposite response of these two systems to the  $\Delta\mu_{H^+}$  is a consequence of the difference in location of the two redox centers. We have shown from theoretical considerations, that a  $\Delta\mu_{H^+}$ , interior negative and alkaline, has an oxidizing effect on a center located at or near the inner surface of the membrane and a reducing effect on a center located at or near the outer surface of the membrane when the electrochemical potential of the electron is at equilibrium throughout the membrane [17]. Since pCMBS only partially protected proline transport activity against inactivation it suggested by analogy with the phosphotransferase system, that the site of reaction of MalNEt might have been a dithiol on the inner surface of the cytoplasmic membrane. Consequently proline transport activity of inverted vesicles was examined. Ferricyanide inhibited proline accumulation and also protected against GSM inactivation in the inverted vesicles confirming the presence of a dithiol near the inner surface of the cytoplasmic membrane which functions in proline transport. These results are analogous to those presented in Fig. 1 and 2 for proline transport into right-side-out vesicles. In addition to demonstrating that dithiols associated with proline transport activity are located on both sides of the cytoplasmic membrane, the results in Fig. 1 and 6 together also indicate that the redox center on the outer surface of the compartment (i.e. either the right-side-out or inverted vesicles) must be in the reduced form before active transport of proline into that compartment can take place. In each case ferricyanide inhibits and dithiothreitol restores the transport activity. In both systems discussed above the polarity of the potential driving the active transport process was interior negative and/or alkaline. Theoretical considerations [17] indicate that the redox center on the outer surface of the compartment should be in the reduced form under such circumstances (see Scheme 1) as was observed. These considerations also indicate that such a potential should shift the redox center on the inside of the compartment into the oxidized state. The simplest way to test this expectation is to use inverted vesicles and establish a potential by oxidation of reduced PMS. Substrate oxidation leads to a potential interior positive and/or acid in inverted vesicles [33]. Consequently redox centers on or near the inner surface of the compartment should now be in the reduced form and those on or near the outer surface should be in the oxidized form. The protection of the proline transport activity against GSM inhibition observed in inverted vesicles (see Fig. 7) when a potential over the membrane was established with reduced PMS confirms that the accessible redox center was in the



Scheme 1. The distribution of dithiol-disulphide in the carrier of L-proline in response to the imposed  $\Delta\psi$ .

oxidized form. These data are analogous to those obtained in similar studies of the glucose transport activity of inverted membrane vesicles [15]. The dithiol-disulphide distributions observed in these studies and their response to potentials of various polarities in right-side-out and inverted vesicles is summarized in Scheme 1.

Although these diagrams illustrate how the redox states respond to the potential, a number of precautionary remarks are in order. The first is that we cannot specify the number of redox centers belonging to a single active carrier. Possibilities would be: (a) a carrier constructed as a fixed channel spanning the membranes with two redox centers, one at each end; (b) carriers constructed as shuttles which do not span the membrane but can rotate or move perpendicular to the plane of the membrane. The redox center or centers of such a protein should be exposed only at one surface at a given time. The latter alternative explains more easily the observations that complete inhibition never seems to result from any of the treatments employed in those experiments reported in Fig. 1–5. Only a given population of the carriers seemed to be accessible to the non-permeable reagents. Those carriers whose redox centers are protected from reaction by virtue of their location, temporarily, at the outer surface of the membrane are the ones responsible for uptake during the subsequent activity measurements. The second precautionary remark concerns the static versus dynamic character of these redox states. It is clear that the dithiol-disulphide interconversions occur when a potential is established over the membrane. However, we cannot determine on the basis of the present data, whether further interconversions occur during the transport step itself.

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